The Metabolism of Nitrate and Nitrite in the Sheep

1. THE REDUCTION OF NITRATE IN THE RUMEN OF THE SHEEP

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A disease of cattle following the ingestion of young oat hay or straw, referred to as 'oat hay poisoning', was reported in the U.S.A. by Newsom, Stout, Thorp, Barber & Groth (1937). Subsequently, Bradley, Eppson & Beath (1939) were able to produce the disease in the laboratory by feeding a steer on the affected hay. Analysis of a number of samples of toxic hay revealed that in all cases nitrate was present in abnormally high concentrations—3·2–7·2% of the dry matter. Bradley, Eppson & Beath (1940) showed that the ingestion of potassium nitrate produced the same symptoms, and simultaneously observed a severe methaemoglobinaemia. It was found that 1 g. potassium nitrate/kg. body weight was a lethal dose, and in a few hours resulted in the conversion of 70–80% of the haemoglobin into methaemoglobin. Sheep were affected in the same way. Since nitrite is known to convert haemoglobin into methaemoglobin, it was concluded that in the alimentary tract of the ruminant nitrate was reduced to nitrite and that the latter gave rise to the methaemoglobinaemia. A similar disorder, 'tribulosis', had been reported many years previously in South Africa (Rimington & Quin, 1933; quoted by Russell, 1944). Symptoms resembling those of 'tribulosis' were produced by dosing sheep with the expressed juice of species of *Tribulus* or with potassium nitrate itself. While the present work was in progress the South African group reported that incubation of untreated rumen contents *in vitro* with nitrate resulted in the formation of nitrite (Sapiro, Hoflund, Clark & Quin, 1949).

The present investigations were designed to provide information concerning (*a*) the reduction of nitrate to nitrite *in vivo* in the rumen of the sheep, and the further reduction of nitrite to ammonia; (*b*) the changes, if any, in the ammonia content of the rumen fluid subsequent to dosing with nitrate; (*c*) the degree of methaemoglobinaemia produced after different amounts of nitrate or nitrite are placed in the rumen, and after the intravenous injection of known amounts of nitrite.

METHODS

The experimental sheep were Oxford Down × Halfbred 3-year-old wethers, fitted with a permanent rumen fistula as described by Phillipson & Innes (1939). They were kept in metabolism cages and fed 3·5 lb. (1·6 kg.) good meadow hay daily, with water *ad lib.* The weights of the sheep remained constant, ±1 kg., throughout the period of study. Urine samples, free from faeces, were collected under toluene. Blood samples were taken from the jugular vein, and clotting was prevented by sodium oxalate.

Samples of rumen contents were withdrawn through the fistula and the material so obtained filtered through muslin. The filtrate is termed rumen liquor (RL). All analyses of RL were carried out on a clear filtrate obtained by the following procedure: 2 ml. saturated lead acetate were added to 10 ml. of RL followed by 0·5 g. decolorizing char-

Table 1. *Recovery of nitrate added to rumen liquor, urine and water*

Material analysed	Nitrate added (mg. NO_3-N/100 ml.)	Colorimetric method		Conway method		Fe/H_2SO_4 reduction	
		Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)
Rumen liquor	0	0·36	—	0·33	—	0·32	—
	10	9·7	93	9·8	95	9·3	90
	20	19·4	95	19·8	97	18·5	91
	30	29·2	96	29·4	97	28·5	94
Urine	0	0·45	—	0·24	—	0·20	—
	20	22·9	112	19·6	97	18·6	92
	40	34·7	85	38·2	95	37·4	93
Water	0	—	—	—	—	—	—
	5	4·95	99	4·90	98	5·05	101
	10	9·85	99	10·00	100	9·73	97

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coal; after shaking, 3 ml. saturated Na_2SO_4 were added to precipitate the excess lead, and the mixture filtered through a Whatman no. 1 filter paper. This procedure yielded a colourless clear filtrate. Urine samples were clarified in the same way, but the solutions were always very faintly coloured. All doses of $NaNO_3$ and $NaNO_2$ were administered to the sheep as 10% (w/v) aqueous solutions, unless otherwise stated.

Nitrite plus nitrate in RL filtrates were determined by the colorimetric phenoldisulphonic acid method (Association of Official Agricultural Chemists, 1947) using a Spekker photoelectric absorptiometer with a spectral violet Ilford filter no. 601 (maximum transmission 430 mμ.). Urine filtrates were unsuitable for the colorimetric method and were therefore analysed by the following volumetric methods. Preformed NH_3 and total nitrite and nitrate were determined by NH_3 estimations before and after reduction either by Devarda's alloy (Conway, 1947) or by powdered Fe and 8% (w/v) H_2SO_4 (Lees & Quastel, 1946). Table 1 shows that the three methods gave comparable results, with recoveries of nitrate added to normal RL consistently above 90%.

Nitrite was estimated by the Griess-Ilosvay method (Association of Official Agricultural Chemists, 1947) using a King photoelectric colorimeter with a green filter (maximum transmission 520 mμ.). Recoveries of nitrite added to RL or urine were within the limits of 91–99% and duplicates agreed ±3%.

Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938) using the Beckman spectrophotometer.

RESULTS

Metabolism of nitrate in the rumen

The nitrate and nitrite concentrations in the rumen of the untreated animal are uniformly low throughout a 24 hr. period. However, the ammonia concentration rises after feeding (McDonald, 1948), a fact which complicates the interpretation of the ammonia values if the animal is dosed with nitrate at the time of feeding.

McDonald (1949), however, has shown that the ammonia content of rumen liquor is relatively constant during the last 8 hr. before feeding. This observation suggested an experimental procedure which would permit an assessment of the ammonia formation from nitrate. The animal was fed at 6 p.m., and samples of RL were collected from 8 a.m. the following morning. This experiment served as a control; it may be seen from Fig. 1 that the ammonia level remained constant, thus confirming McDonald's observation. The procedure was repeated save that 12 g. sodium nitrate were introduced into the rumen 16 hr. after feeding.

The results (Fig. 1) show that there was a rapid fall in the nitrate, coincident with the production of nitrite which, however, disappeared during the succeeding 5 hr. In the control experiment the ammonia concentration remained constant throughout, whilst after the addition of nitrate there was a definite rise lasting 5–7 hr. This may be due to a stimulation of ammonia production by the nitrate or a conversion of the nitrate to ammonia. The latter is the more probable, since only a small proportion of the nitrate is accounted for in the urine or blood, and, as will be shown later, it cannot all be absorbed as nitrite, for such a quantity would be far in excess of the lethal dose. In this experiment excretion in the urine accounted for less than 8% of the nitrate introduced into the rumen: the nitrite content was very low, and the ammonia values variable.

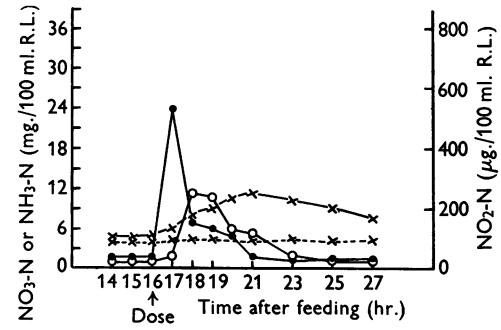

Fig. 1. Nitrate, nitrite and ammonia in RL after placing 12 g. $NaNO_3$ in the rumen, 16 hr. after feeding. ×- - -×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

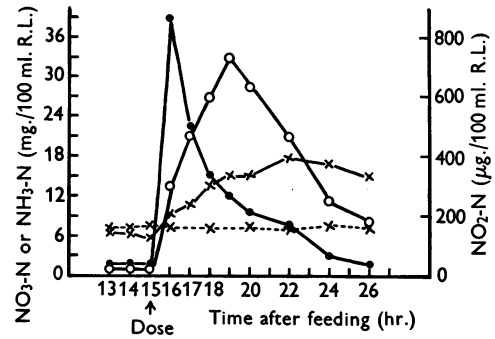

Fig. 2. Nitrate, nitrite and ammonia in RL after introducing 25 g. $NaNO_3$ into the rumen, 15 hr. after feeding. ×- - -×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

The changes in the nitrate, nitrite and ammonia of the RL, following a dose of sodium nitrate sufficient to produce a severe methaemoglobinaemia, were next investigated, 25 g. sodium nitrate being given to the sheep 15 hr. after feeding. A control experiment was carried out in which no nitrate was administered. The results are presented in Fig. 2. The methaemoglobin content of the blood had

reached its maximum 7 hr. after dosing and amounted to 63 % of the total haemoglobin.

The results show the same general tendencies as in Fig. 1, when half this quantity of sodium nitrate was administered, but the increase in nitrite was much more marked. The nitrate, nitrite and ammonia concentrations in the RL were constant in the control experiment. The nitrate concentration 1 hr. after dosing was 39 mg. NO_3-N/100 ml. RL (25 g. sodium nitrate in 6 l. RL is equivalent to 68·6 mg. NO_3-N/100 ml. RL). Whilst the nitrate concentration fell rapidly there was still an appreciable amount present 6 hr. after dosing. The nitrite concentration rose steadily for 4 hr. and fell uniformly almost to normal during the next 6 hr., but there was still a considerable amount of nitrite (460 μg. NO_2-N/100 ml. RL) present in the RL 7 hr. after dosing, at which time the methaemoglobin content of the blood was at its maximum. There was a pronounced rise in the ammonia concentration, which presumably accounted for a considerable proportion of the nitrate disappearing at the later stages. Only a small proportion (4 %) of the nitrate was lost in the urine.

Nitrate and methaemoglobin formation

A series of experiments was carried out in which the methaemoglobin content of the blood was determined after increasing quantities of sodium nitrate (0–25 g.) had been placed in the rumen. 11–13 % (w/v) of the blood. The results are given in Fig. 3.

As the quantity of sodium nitrate administered was increased from 12 to 22·5 g. the extent of the methaemoglobin production gradually became greater, and the time between administration and the maximum value attained became longer. When the dose was increased to 25 g. sodium nitrate there was a sharp rise in the maximum methaemoglobin. This may be due to the fact that above a certain concentration of nitrate the rate of reduction of nitrite to ammonia becomes limiting, and in consequence nitrite accumulates in the rumen. This nitrite passes into the blood in increasing concentration and gives rise to a severe methaemoglobinaemia.

Nitrite and methaemoglobin formation

A further series of experiments was carried out in which sodium nitrite was placed in the rumen. The quantity administered was increased daily as with nitrate. Samples of blood were collected and methaemoglobin estimated (Fig. 4). The administration of less than 5 g. sodium nitrite (5 % (w/v) solution) had but little effect on the methaemoglobin content of the blood. There was a significant methaemoglobinaemia when 7 or 9 g. sodium nitrite were placed in the rumen, with a further increase in the maximum methaemoglobinaemia attained when the dose was increased to 10 g. sodium nitrite. The

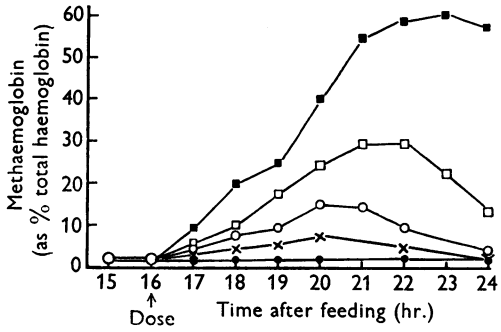

Fig. 3. Methaemoglobin production when various amounts of $NaNO_3$ are placed in the rumen, 16 hr. after the sheep was fed. ●—●, Exp. 3, 0 g. $NaNO_3$; ×—×, Exp. 4, 12 g. $NaNO_3$; ○—○, Exp. 5, 17·5 g. $NaNO_3$; □—□, Exp. 6, 22·5 g. $NaNO_3$; ■—■, Exp. 7, 25 g. $NaNO_3$.

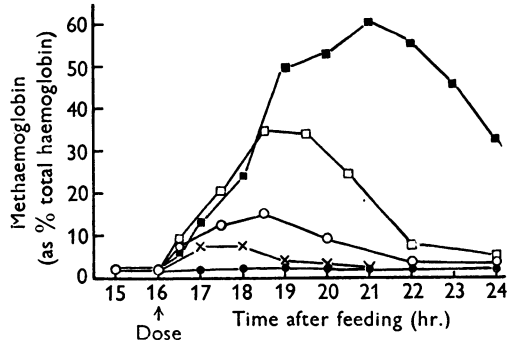

Fig. 4. Methaemoglobin production when various amounts of $NaNO_2$ are placed in the rumen, 16 hr. after the sheep was fed. ●—●, Exp. 10, 1 g. $NaNO_2$; ×—×, Exp. 11, 5 g. $NaNO_2$; ○—○, Exp. 12, 7 g. $NaNO_2$; □—□, Exp. 13, 9 g. $NaNO_2$; ■—■, Exp. 14, 10 g. $NaNO_2$.

A different quantity was administered daily, and control experiments, in which no nitrate was administered, were carried out at the beginning and at the end of the series. The results of these control experiments showed excellent agreement. After dosing, samples of blood were collected at intervals over the succeeding 8 hr. period and analysed for total haemoglobin and methaemoglobin. The haemoglobin contents of all samples lay within the range of

length of time between the administration and the peak values again became greater as the dose was raised.

The results of the analysis of samples of RL taken at intervals after 10 g. sodium nitrite were placed in the rumen are presented in Table 2, and the results obtained following an equivalent dose of sodium nitrate, are included for comparison. The nitrite concentration remained above 500 μg. NO_2-N/100 ml.

Table 2. NH_3-N *and* NO_2-N *in rumen liquor, and methaemoglobin in blood, after placing* 10 *g.* $NaNO_2$ *or* 12 *g.* $NaNO_3$ *in the rumen*

(The dose was given 16 hr. after feeding.)

Time after feeding (hr.)	Exp. 16, 10 g. $NaNO_2$ in rumen			Exp. 4, 12 g. $NaNO_3$ in rumen		
	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb
15	4·5	12·5	2·0	4·0	10	1·5
16	4·6	10·0	2·3	4·0	11	1·7
17	5·4	17,500	13	4·9	20	2·5
18	7·0	8,100	24	9·8	248	4·0
19	9·4	2,750	49	10·8	232	5·5
20	9·9	1,250	53	10·9	122	8·0
21	9·3	570	61	11·1	100	—
23	8·1	120	46	10·2	26	4·9
25	5·7	30	32	9·0	18	—
27	4·9	15	—	8·0	16	—

RL for 5 hr. after the administration of the sodium nitrite while the concentration after administration of 12 g. sodium nitrate was negligible by comparison. However, the changes in ammonia concentrations were very similar, supporting the concept that nitrate is reduced in the rumen to ammonia, with nitrite as an intermediate. Nitrite and nitrate excretion was negligible.

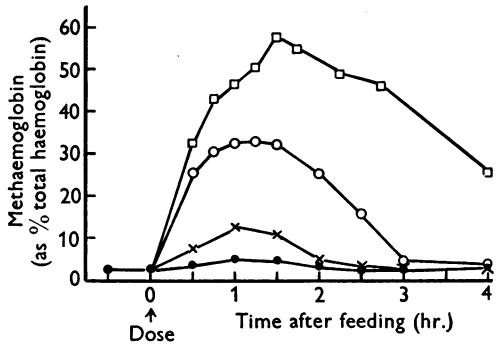

Fig. 5. Methaemoglobin production on the intravenous injection of $NaNO_2$. ●—●, Exp. 17, 0·5 g. $NaNO_2$; ×—×, Exp. 18, 1 g. $NaNO_2$; ○—○, Exp. 19, 1·5 g. $NaNO_2$; □—□, Exp. 20, 2 g. $NaNO_2$.

For comparison a series of experiments was carried out in which sodium nitrate was injected into the jugular vein in order to determine the amount of methaemoglobin produced by the interaction of known amounts of nitrite and the haemoglobin of the circulating blood. Samples of blood and urine were collected at intervals. The nitrite excreted in the urine was again negligible and in no case accounted for more than 1–2% of the sodium nitrite given. A conversion of 60% of the blood haemoglobin into methaemoglobin resulted when 2 g. sodium nitrite were injected (Fig. 5). The rise of methaemoglobin to its maximum was rapid and there was also a sharp increase in the methaemoglobin maximum as the dose was increased (Fig. 6). This is to be expected since in this experiment the nitrite and haemoglobin were brought into immediate contact, and the rate of methaemoglobin formation was independent of the rates of reduction processes or of passage through the rumen wall.

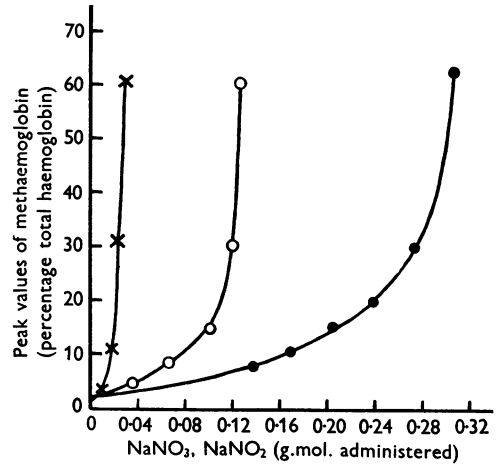

Fig. 6. Peak values of methaemoglobin plotted against dosage. ●—●, nitrate placed in the rumen; ○—○, nitrite placed in the rumen; ×—×, nitrite injected intravenously.

The peak methaemoglobin values produced on introducing various amounts of nitrate or nitrite into the rumen are compared in Fig. 6 with those resulting from the intravenous injection of nitrite. When less than 0·25 mol. nitrate or 0·11 mol. nitrite was added to the rumen, there was an approximately linear relationship between the amount administered and the extent of the methaemoglobinaemia. Greater quantities resulted in a disproportionately large formation of methaemo-

globin. This suggests that above a certain concentration of nitrite the rate of reduction of nitrite to ammonia is limited, and not as rapid as the reduction of nitrate to nitrite. In both cases nitrite would thus accumulate in the rumen, and presumably pass into the blood stream.

DISCUSSION

It is evident that nitrate is reduced to ammonia by the micro-organisms in the rumen of the sheep, and that nitrite is an intermediate in this reaction. The nitrite accumulates under certain conditions, and may lead to a production of methaemoglobin in the blood. A dose of 25 g. sodium nitrate or 10 g. sodium nitrite introduced into the rumen of the sheep (60 kg.) produces the same degree of methaemoglobinaemia (60 % of the total haemoglobin), as 2 g. sodium nitrite injected intravenously. The amount of nitrite injected intravenously may be expressed quantitatively in relation to the haemoglobin converted into methaemoglobin. The live weight of the sheep was 60 kg. and the blood haemoglobin level was 12 % (w/v). It is assumed that the sheep has a blood volume equivalent to 10 % of the live weight and that the iron content of the haemoglobin is 0·34 %. Knowing that 2 g. sodium nitrite administered intravenously converted 60 % of the haemoglobin to methaemoglobin it can be shown that approximately 4 mol. sodium nitrite are required to produce 1 mol. methaemoglobin. These figures are relevant to *in vivo* conditions and to the particular sheep employed.

The fact that 10 g. sodium nitrite introduced into the rumen has a similar effect suggested that only 20 % of the nitrite passed to the blood stream. Sodium nitrate (25 g.) placed in the rumen results in a similar degree of methaemoglobinaemia, but there are probably so many factors governing the methaemoglobin production in this instance that it is only possible to say that this is effectively equivalent to 10 g. sodium nitrite.

The bulk of the nitrate placed in the rumen is undoubtedly reduced to ammonia, and nitrite is an intermediate in this reaction. This nitrite can pass, to a greater or less extent, into the blood stream, where it leads to the conversion of part of the haemoglobin to methaemoglobin. Increasing the dose of nitrate to 22·5 g. sodium nitrate or more, led to a marked accumulation of nitrite in the rumen and a sharp increase in the extent of methaemoglobinaemia. It is concluded that at this level the rate of reduction of nitrite to ammonia becomes a limiting factor in the removal of nitrite, whereas further added nitrate is still reduced to nitrite. In the same way when nitrite is introduced into the rumen the reduction to ammonia becomes limited above a certain concentration of nitrite. In both cases, following doses of nitrate or nitrite greater than certain critical amounts the sheep will suffer from a methaemoglobinaemia that rapidly becomes more severe with larger doses, as was found experimentally (Fig. 6).

It is possible to correlate the peak methaemoglobin values with the changes in the nitrate, nitrite and ammonia in the rumen liquor after administration of nitrate. When 12 g. sodium nitrate was administered to the sheep (Fig. 1) the nitrate concentration fell off rapidly at first and then more slowly, while the ammonia values rose steadily for 5 hr. Nitrite was present in appreciable amount only for a short period during the initial rapid fall in the quantity of the nitrate present. It is possible that most of the ammonia, produced initially, enters the normal synthetic mechanisms in the rumen. The ammonia produced later would possibly be less utilized in this way owing to saturation of this mechanism and the rise in the concentration would be greater in proportion to the nitrate disappearing, on the whole fitting in with the observed steady rise in ammonia over several hours. This situation is still more obvious when 25 g. sodium nitrate were given to the sheep. Since the amount of nitrate present was still appreciable 5–6 hr. after administration the ammonia values increased over a longer period and to a higher level. The quantity of nitrite present in this case was greater, and persisted in the rumen for several hours. The extent of the methaemoglobinaemia increased to a peak of 60 % of the total haemoglobin at 7 hr. after dosing with nitrate.

SUMMARY

1. Nitrate introduced into the rumen of the sheep is reduced to ammonia.

2. Nitrite is an intermediate in this reaction and may accumulate under certain conditions and lead to a partial conversion of blood haemoglobin to methaemoglobin.

3. Sodium nitrate (25 g.) or sodium nitrite (10 g.) placed in the rumen, or 2 g. sodium nitrite injected intravenously, result in a methaemoglobinaemia corresponding to 60 % conversion of the total haemoglobin.

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The Use of Radioactive Isotopes in Immunological Investigations

5. QUANTITATIVE ASPECTS OF THE ANTIBODY-ANTIGEN REACTION

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The reaction between antigen and antibody which results in precipitation has been studied quantitatively by Heidelberger, Marrack, Haurowitz, Kabat, Hooker and Boyd, and many other investigators (see reviews by Heidelberger, 1939, 1946; Marrack, 1938; Boyd, 1947; Kabat & Mayer, 1948; Wormall, 1948). These investigations have been based mainly on the determination of small amounts of nitrogen in the precipitates. The amount of antibody can be readily calculated from these determinations if the antigen contains little or no nitrogen (e.g. polysaccharides) or alternatively, the amounts of both antigen and antibody can be calculated, if the antigen contains some special group or element (e.g. dyestuff, iron, copper, etc.) which can be determined separately. Few natural proteins, however, contain a suitable label, and the introduction of foreign groups, leading to the production of unnatural proteins or protein complexes, is frequently undesirable. When unlabelled protein antigens are used, the analysis of the specific precipitates for both antigen and antibody is complicated, and may sometimes be impossible.

We have used antigens and antibodies labelled with radioactive isotopes to study the precipitin test and certain other serological reactions. At first our choice of antigen was limited to biologically prepared labelled proteins such as lipovitellin and vitellin (Francis & Wormall, 1948), and chemically altered proteins such as phosphorylated or mustard gas sulphone-treated proteins containing appreciable quantities of introduced phosphorus or sulphur (Boursnell, Dewey, Francis & Wormall, 1947; Banks, Boursnell, Dewey, Francis, Tupper & Wormall, 1948). With an improved supply of certain carrier-free radio-isotopes it later became possible, however, to use such small amounts of the element that the protein molecule could be adequately labelled without significant change in the properties of the protein other than the acquirement of radioactivity. The experiments described here were carried out in order (*a*) to obtain information about the best methods for labelling protein antigens and antibodies with radio-isotopes for serological investigations, and (*b*) to use these labelled compounds for quantitative studies on certain precipitin reactions. In most of these experiments chemically altered protein antigens have been used, e.g. those labelled with 8–12% radio-iodine; but where antibodies have been labelled with ^{131}I this has been effected with small amounts of radio-iodine of high specific activity. Preliminary notes of a few of these investigations have been published elsewhere (Boursnell *et al.* 1947; Banks, Francis, Mulligan & Wormall, 1949, 1950).

EXPERIMENTAL

Methods

Precipitin reactions. Since we usually varied the technique of these tests to suit the particular experiment undertaken, only the general details will be given here; exact details, where they are required, are given in the appropriate tables or parts of the text. The antigen and antibody solutions, plus